

EXPRESS MAIL NO. ET593400038US
Attorney Docket No.: 20093-000810US

PATENT APPLICATION

**METHOD TO INCREASE CLASS I PRESENTATION OF EXOGENOUS
ANTIGENS BY HUMAN DENDRITIC CELLS**

Inventor(s):

Michael L. Salgaller, a United States citizen, residing at
12517 80th Place NE
Kirkland, Washington 98034

Alton L. Boynton, a United States citizen, residing at
2420 274th Avenue NE
Redmond, Washington 98053

Assignee:

Northwest Biotherapeutics, Inc.
21720 23rd Drive S.E., Suite 100
Bothell, Washington 98021

Entity: Small Entity

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, California 94111-3834
Tel: 206-467-9600

METHOD TO INCREASE CLASS I PRESENTATION OF EXOGENOUS ANTIGENS BY HUMAN DENDRITIC CELLS

CROSS REFERENCES TO RELATED APPLICATIONS

5

This application claims priority to United States Provisional Patent Application No. 60/203,758, filed May 12, 2000, incorporated herein by reference in its entirety.

10

BACKGROUND OF THE INVENTION

It is well established that the immune system can function to kill tumor cells, including both primary and metastatic cancer cells. Indeed, evidence that the immune system recognizes the presence of neoplastic cancerous cells is supported by the existence of infiltrating lymphocytes in tumor tissues (Haskill et al., *Contemp. Top. Immunobiol.* 8:107-170 (1978); Vose and Moore, *Semin. Hematol.* 22:27-40 (1985)). Despite the presence of immune cells, tumors often prevail and not only survive but metastasize to distant sites with unrestricted growth.

Recent advances in the understanding of T cell activation and recognition of target cells has permitted some progress in the development of T cell mediated cancer immunotherapy (Schwartz, *Cell* 71:1065-1068 (1992); Pardoll, , *Curr. Opin. Immunol.* 4:619-623 (1992)).

25

The immune system develops from a single multi-potential progenitor cell into the major subgroups of lymphoid and myeloid cells. Lymphoid cells are comprised of B cells and T cells. Myeloid cells include macrophages, monocytes and neutrophils. Immune cells are capable of circulating and seeking out foreign antigens and eliminating them.

30

In the lymphoid subgroup, the immune response leads to the activation of helper T cells (T_H) which are positive for expression of the surface molecule $CD4^+$ and of cytotoxic T cells (T_C) which are surface positive for the molecule $CD8^+$. T cells are activated through interaction with antigen presenting cells (APC) that express major histocompatibility

(MHC)-class I or -class II molecules associated with antigenic fragments. The associated antigenic amino acid sequences are specifically derived from the processed antigen.

APCs use two alternative methods to present antigens depending on the source of the antigen. Exogenous, soluble antigen is endocytosed into vacuoles and degraded by low pH. The peptide fragments that result are then directed to MHC-class II proteins and presented on the cell surface. Presentation on MHC-class I requires that antigens are degraded in the cytosol and transported by the TAP transporter system into the endoplasmic reticulum. Typically this requires that the antigen be in the cytosol, for example in the case of a viral infection or by cellular translation, and the resultant peptides then associate with MHC-class I.

The antigen-MHC complex is recognized by the specific T cell receptor which recognizes the antigen, and the CD4 and CD8 surface molecules. CD4 and CD8 interact with conserved regions of only one class of MHC each. Whereas MHC-class II is recognized by T_H cells due to interaction with CD4, MHC-class I presentation is restricted to activating T_C cells through interaction with CD8.

The activation of naive or primed T cells follows a defined mechanism. Endogenous antigen is presented on MHC-class I and soluble exogenous antigen is presented on MHC-class II by APCs. The MHC-class I or MHC-class II antigen complexes interact with CD8 or CD4 respectively and also interact with the T cell receptor specific for the antigen. Upon this specific interaction, secondary molecules such as β -microglobulin and CD28 trigger activation of the T cells which then exert the appropriate immune response.

The sensitized or “primed” $CD4^+$ T cells produce chemokines that participate in the activation and recruitment of B cells as well as various T cell subsets. The sensitized $CD8^+$ T cells increase in numbers in response to lymphokines and are capable of destroying any cells that express the specific antigenic fragments associated with matching MHC-class I molecules (Jondal et al., *Immunity* 5:295-302 (1996)).

Tumor infiltrating lymphocytes are evidence that cancerous tumors induce $CD8^+$ CTL capable of eradicating cells expressing cancer associated or cancer specific antigens, thereby limiting the progression of tumor spread and disease development.

However, tumors frequently grow and metastasize, overcoming this natural immunity. Various methods for immunotherapy directed to a number of particular cancers have been suggested to enhance this natural immune response, however, the primary difficulty has been

inducing APCs to present soluble human tumor associated or tissue specific antigens via MHC-class I. Recent experiments have demonstrated in murine systems, that activation of CTLs *in vitro* can confer a potent protection from growth of syngeneic tumors *in vivo* (Fields et al., *Proc. Natl. Acad. Sci. USA* 95:9482-9487 (1998)). However, experiments in the
5 murine immune system are not completely predictive of human immune responses. To date there are no therapeutic methods that successfully elicits an effective human CTL immunotherapeutic response against primary or metastatic cancer using APCs incubated with a soluble protein or proteinaceous antigen.

Antigen presenting cells (APCs) are particularly important in eliciting an
10 effective immune response. By definition, APCs not only present antigens to T cells with antigen-specific T cell receptors, but provide all the signals necessary for T cell activation. The signals necessary for T cell activation are incompletely defined, but probably involve a variety of cell surface molecules as well as cytokines or growth factors. Further, the factors necessary for the activation of naive or unprimed T cells may be different from those required for the re-activation of previously primed memory T cells. The ability of APCs to both present antigens and deliver signals for T cell activation is commonly referred to as an accessory cell function. Although monocytes and B cells have been shown to be competent APC, their antigen presenting capacities *in vitro* appear to be limited to the re-activation of previously sensitized T cells. Hence, they are not capable of directly activating functionally naive or unprimed T cell populations.
15
20

The term “dendritic cells” refers to a diverse population of morphologically similar cell types found in a variety of lymphoid and non-lymphoid tissues (Steinman, *Ann. Rev. Immunol.* 9:271-296 (1991)). These cells include lymphoid dendritic cells of the spleen, Langerhans cells of the epidermis, and veiled cells in the blood circulation. Although they
25 are collectively classified as a group based on their morphology, high levels of surface MHC class II expression, and absence of certain other surface markers expressed on T cells, B cells, monocytes, and natural killer cells, it is presently not known whether dendritic cells derive from a common precursor or can all function as APCs in the same manner. Dendritic cells are the most potent APCs of the immune system capable of stimulating primary T and B
30 lymphocyte responses. (Banchereau et al., *Nature* 392:245-252 (1998)).

Studies have described methods for the isolation and expansion of human DCs from many sources, including, from human peripheral blood. (Macatonia et al., *Immunology*

74:399-406 (1991); O'Doherty et al., *J. Exp. Med.* 178:1067-1078 (1993) (isolation); and
Markowicz et al., *J. Clin. Invest.* 85:955-961 (1991); Romani et al., *J. Exp. Med.* 180:83-93
(1994); Sallusto et al., *J. Exp. Med.* 179:1109-1118 (1994); Bernhard et al., *Cancer Res.*
55:1099-1104 (1995) (expansion)). PCT Publication WO 94/02156 describes a method for
isolating human DCs to present antigens to induce antigen specific T cell-mediated
responses. Adoptive cellular immunotherapy and use of the isolated DCs against infectious
diseases and cancer are mentioned.

Clinical trials have been initiated relating to the use of dendritic cells pulsed
with a relevant antigen against melanoma (Nestle, F.O., et al., *Nat. Med.* 4:328-332 (1998));
10 against B cell lymphoma (Hsu, F.J., et al., *Nat. Med.* 2:52-58 (1996)); and against prostate
cancer (U.S. Patent No. 5,788,963 to Murphy et al; Murphy et al., *Prostate* 29:371-380
(1996); Salgaller, et al., *Prostate* 35:144-151 (1998)).

Exogenous antigen processing results in antigen presentation on MHC-class II,
whereas the endogenous processing pathway utilizes MHC-class I (Jondal et al., *Immunity*,
15 5:295-302 (1996)). The activation of T_H cells by the MHC-class II presentation of antigen
has had therapeutic success, but experiments in murine models suggest a significantly more
potent cancer protection would result if presentation were by MHC-class I. However,
success in a murine model is not always predictive of success using human cells and there
have been no reports of MHC-class I presentation of soluble exogenous antigens by human
20 APCs.

Although there is a small subgroup of soluble antigens that are presented on
MHC-class I, including some bacterial and viral antigens, a method of reliably inducing
MHC-class I presentation of many antigens, including, for example, exogenous soluble
human tumor associated or tissue specific antigens has not been reported. Techniques have
25 been developed to create fusion proteins of soluble antigens that are presented on MHC-class
I and antigens of interest, however this process requires significant time and molecular
manipulations to implement effectively. MHC-class I processing of an exogenous antigen
could potentially represent a significant improvement in current immunotherapies.

Prostate cancer is the most common form of cancer currently diagnosed in
30 American men. It is second only to lung cancer as the leading cause of cancer deaths among
adult males. Nearly a third of all newly diagnosed prostate cancer patients present with
metastatic or locally advanced disease. At present, available therapies for metastatic disease,

including hormonal, chemotherapeutic and radiation approaches, have not achieved curative potential in a significant percentage of patients. For those with localized carcinoma, prostatectomy and radiotherapy, the current standards of treatment result in failure rates of between 20 and 50%. The options for these primary treatment failures, as with those with progressed disease, are few in number and limited in clinical benefit.

With the elucidation of the mechanisms involved in immune recognition, new and exciting strategies in anti-cancer therapeutics have become available. Of particular promise are those cancer vaccines which utilize antigen presentation by dendritic cells (DCs) to elevate the anti-tumor response. However, recent work has shown that it is important to enhance the delivery of soluble, exogenous, tumor-associated antigens into the major histocompatibility complex (MHC) class I processing compartment of DCs to maximize the cellular immune response. The present invention addresses these and other needs in the art.

SUMMARY OF THE INVENTION

The present invention provides methods, and compositions, for dendritic cell activation of T cells in immunotherapeutic responses against primary or metastatic cancer. The DCs obtained from human donors are administered to a cancer patient in need thereof, following exposure to a soluble tissue associated, tissue specific, tumor associated, or tumor specific antigen in combination with an adjuvant that increases the MHC class-I associated cytotoxic T cell response *in vivo* as compared to the response induced by antigen alone. Alternatively, the antigen used for exposure to the DCs can be a fragment of the tissue associated, tissue specific, or tumor associated antigen. In one embodiment, the DCs are exposed simultaneously to the adjuvant and the soluble tissue associated, tissue-specific or tumor antigen, or antigenic fragments thereof. This response includes helper T cell (T_H) and cytotoxic T cell (T_C) activation. Alternatively, human T cells are cultured *in vitro* with the foregoing DCs and the *in vitro* activated T cells are subsequently administered to a cancer patient in need thereof.

In one embodiment of the invention, bacillus Calmette Guerin (BCG), *Mycobacteria bovis*, is used as an adjuvant with an antigen, *i.e.*, a soluble tumor or tissue specific protein antigen or antigenic fragment thereof to obtain MHC-class I processing. Exogenous antigen is normally processed by the MHC-class II compartment in antigen

presenting cells (APC) and endogenous antigens are processed by the MHC-class I compartment. Surprisingly, the present inventors have found that when DCs are pulsed with a soluble antigen, including human tumor antigen or tissue specific antigens with an adjuvant such as BCG, enhancement of MHC-class I presentation occurs. Therefore, the presence of 5 an adjuvant such as BCG typically increases DC soluble tumor antigen processing in the MHC-class I compartment and correspondingly, activates a higher percentage of CD8⁺ T cells when compared to individuals administered the antigen alone.

In another embodiment, DCs are exposed to soluble antigen, including viral, bacterial, tissue, tissue specific, tumor associated, or tumor specific antigen in the presence of 10 a combination of BCG and a bacterial exotoxin, such as, lipopolysaccharide (LPS). According to one embodiment a prostate tumor cell lysate recovered from a surgical specimen can be used as antigen. For example, a sample of a prostate cancer patient's own tumor, obtained at biopsy or at surgical resection, can be used to provide a cell lysate for 15 antigen. Further, purified prostate specific membrane antigen (PSMA, also known as PSM antigen), which specifically reacts with monoclonal antibody 7E11-C.5 can be used as an antigen. Additional antigens include antigenic fragments of a tissue associated, tissue specific, tumor associated or tumor specific protein antigen, *i.e.*, such as PSMA, prostate mucin antigen, prostate specific antigen, prostate acid phosphatase (PAP), PD41 antigen, and the like. According to one embodiment an antigenic peptide having the amino acid sequence 20 Leu Leu His Glu Thr Asp Ser Ala Val (SEQ ID NO: 1)(designated PSM-1) which corresponds to amino acid residues 4-12 of PSMA can be used as antigen. Additionally, an antigenic peptide having an amino acid sequence Ala Leu Phe Asp Ile Glu Ser Lys Val (SEQ ID NO: 2) (designated PSM-2), which corresponds to amino acid residues 711-719 of PSMA can be used as antigen.

According to another embodiment, an antigenic peptide selected from 25 antigenic peptide fragments of PSM matched to a binding motif of a specific haplotype. According to an additional embodiment, the peptides are selected to be presented by DCs to activate T cells of a patient which matched the haplotype indicated for each peptide of PSA and which have been matched to a binding motif of a specific haplotype.

In an alternative embodiment, the MHC class-I antigen loaded DCs, described 30 *supra*, are incubated *in vitro* with primed or unprimed T cells to activate the relevant T cell responses. The activated T cells are subsequently administered to a patient, *i.e.*, a cancer for

immunotherapy. In either case, the DCs are advantageously used to elicit an immunotherapeutic growth inhibiting response against, for example, an infection or a primary or metastatic human cancer. In particular, the human cancer is prostate cancer.

In another embodiment, the invention provides a method for producing a tumor cell proliferation growth inhibiting response, which comprises administering, to a cancer patient in need thereof, an effective amount of activated T cells, in which the T cells were activated *in vitro*. The *in vitro* activation includes exposure of human dendritic cells to a tissue associated, tissue specific, tumor associated, tumor specific antigen or antigenic fragments thereof in combination with BCG, either in combination with or without LPS, to enhance MHC-class I processing. In a further embodiment, the invention provides a method for producing a tumor growth or cancer cell proliferation inhibiting response, which comprises administering, to a cancer patient in need thereof, an effective amount of human dendritic cells, exposed *in vitro* to a tissue associated, tissue specific, tumor associated or tumor specific antigen or an antigenic fragment thereof in combination with BCG, in combination with or without LPS, such that after administration, the human DCs elicit a predominately CD8⁺ T cell immune response or augment an existing immune response against the tumor or cancer cells.

Antigens useful for the methods and compositions of the invention include but are not limited to; soluble extracts of tumor cells from a patient biopsy, soluble extracts from tumor cells obtained during surgical resection, tumor specific antigens, tissue associated or tissue specific antigens relevant to the tumor type, recombinant purified tumor antigens, recombinant purified tissue associated or tissue specific antigens, and the like, as set forth herein.

The present invention, further provides compositions comprising isolated human dendritic cells exposed to an adjuvant and a relevant antigen(s) in a particular embodiment, where the dendritic cells are cryopreserved isolated human dendritic cells and extended life span human dendritic cells which are useful for eliciting immunotherapeutic responses against primary and/or metastatic cancer.

BRIEF DESCRIPTION OF THE FIGURES

5 The present invention may be more fully understood by reference to the
following detailed description of the invention, illustrated examples of specific embodiments
of the invention and the appended figures.

10 Fig. 1A through Fig. 1C depict the activation of T cells from prostate cancer
patients by autologous (Fig. 1A) or allogeneic (Fig. 1B and Fig. 1C) dendritic cells
previously loaded by pulsing with LNCaP-derived prostate specific membrane antigen
PSMA and either BCG, or BCG and LPS, or the T cells were pulsed with dendritic cells
osmotically loaded with PSMA alone. Day 18 cultured T cells from Patient 92 were washed
and added to 96-well plates at 5×10^4 cells per well in duplicate. Autologous DCs (Fig. 1A),
or allogeneic DCs from Patient 105 (Fig. 1B) and patient I.T. (Fig. 1C), osmotically loaded
with PSMA (open bars) or ovalbumin (OVA; hatched bars) (or left untreated; crossed bars)
were added to the T cells at 5×10^4 cells per well to test for activation as measured by cytokine
production. After 24 hours incubation, 150 μ l of supernatant was removed from each culture
well and the amount of IFN γ present measured by ELISA and plotted against the y-axis.
20

15 Fig. 2A through Fig. 2C depict the specific reactivity of T cells activated *in vitro*, including both CD8 $^{+}$ and CD4 $^{+}$ T cell groups. Day 25 cultured T cells from Patient 105
were washed and added to 96-well plates at 5×10^4 cells per well in duplicate. DCs were
pulsed with antigen (PSMA or OVA) and either (Fig. 2A) BCG, (Fig. 2B) BCG + LPS.
25 Alternatively, PSMA or OVA were osmotically loaded (Fig. 2C). Autologous DCs pulsed
with PSMA (DC+PSMA), OVA (DC+OVA), or unpulsed (DC alone) were added to Patient
105 T cells at 5×10^4 DCs per well. Effector cells were incubated with either saline (No
mAb; open bars), or 1 μ g/ml anti-CD8 mAb (hatched bars), or 1 μ g/ml anti-CD4 mAb
(crossed bars), in duplicate wells. IFN γ production was measured as in Fig. 1.
30

Fig. 3A through Fig. 3C depict dose dependent effects of dendritic cells
activated *in vitro* with soluble PSMA combined with BCG, or with BCG and LPS on T cells.

T cells from prostate cancer Patient 105 were activated by autologous dendritic cells previously loaded with serial dilutions of PSMA derived from LNCaP cells. ELISAs were performed to assess IFN γ secretion. Day 32 or 39 (Fig. 3C) cultured cells were washed and added to 96-well plates at 5×10^4 cells per well in duplicate. Autologous DC pulsed with either PSMA (open bars), OVA (hatched bars), or unpulsed (crossed bars) were added to the T cells at 5×10^4 cells per well with or without BCG or BCG plus LPS. BCG, day 32 of culture (Fig. 3A); BCG + LPS, day 32 of culture (Fig. 3B); BCG, day 39 of culture (Fig. 3C). After 24 hours incubation, 150 μ l of supernatant was removed from each culture and the amount of IFN γ present was measured by ELISA.

10

Fig. 4A and Fig. 4B demonstrate the stimulation of lytic activity for antigen-specific targets of T cells from prostate cancer patients stimulated *in vitro* with PSMA-expressing DCs. Different ratios of effector (E) (*i.e.*, T cells) to target (T) (*i.e.*, autologous dendritic cells) (E:T) were incubated for four hours. The autologous DCs, osmotically loaded with PSMA (●) or OVA (■), or untreated (▲), were radiolabeled with ^{111}In . Percent lysis was calculated using the following formula: [(experimental release - spontaneous release)/(maximum release - spontaneous release)] \times 100. Patient I.T., day 32 of culture (Fig. 4A). Patient 92, day 39 of culture (Fig. 4B).

20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
130
135
140
145
150
155
160
165
170
175
180
185
190
195
200
205
210
215
220
225
230
235
240
245
250
255
260
265
270
275
280
285
290
295
300
305
310
315
320
325
330
335
340
345
350
355
360
365
370
375
380
385
390
395
400
405
410
415
420
425
430
435
440
445
450
455
460
465
470
475
480
485
490
495
500
505
510
515
520
525
530
535
540
545
550
555
560
565
570
575
580
585
590
595
600
605
610
615
620
625
630
635
640
645
650
655
660
665
670
675
680
685
690
695
700
705
710
715
720
725
730
735
740
745
750
755
760
765
770
775
780
785
790
795
800
805
810
815
820
825
830
835
840
845
850
855
860
865
870
875
880
885
890
895
900
905
910
915
920
925
930
935
940
945
950
955
960
965
970
975
980
985
990
995
1000
1005
1010
1015
1020
1025
1030
1035
1040
1045
1050
1055
1060
1065
1070
1075
1080
1085
1090
1095
1100
1105
1110
1115
1120
1125
1130
1135
1140
1145
1150
1155
1160
1165
1170
1175
1180
1185
1190
1195
1200
1205
1210
1215
1220
1225
1230
1235
1240
1245
1250
1255
1260
1265
1270
1275
1280
1285
1290
1295
1300
1305
1310
1315
1320
1325
1330
1335
1340
1345
1350
1355
1360
1365
1370
1375
1380
1385
1390
1395
1400
1405
1410
1415
1420
1425
1430
1435
1440
1445
1450
1455
1460
1465
1470
1475
1480
1485
1490
1495
1500
1505
1510
1515
1520
1525
1530
1535
1540
1545
1550
1555
1560
1565
1570
1575
1580
1585
1590
1595
1600
1605
1610
1615
1620
1625
1630
1635
1640
1645
1650
1655
1660
1665
1670
1675
1680
1685
1690
1695
1700
1705
1710
1715
1720
1725
1730
1735
1740
1745
1750
1755
1760
1765
1770
1775
1780
1785
1790
1795
1800
1805
1810
1815
1820
1825
1830
1835
1840
1845
1850
1855
1860
1865
1870
1875
1880
1885
1890
1895
1900
1905
1910
1915
1920
1925
1930
1935
1940
1945
1950
1955
1960
1965
1970
1975
1980
1985
1990
1995
2000
2005
2010
2015
2020
2025
2030
2035
2040
2045
2050
2055
2060
2065
2070
2075
2080
2085
2090
2095
2100
2105
2110
2115
2120
2125
2130
2135
2140
2145
2150
2155
2160
2165
2170
2175
2180
2185
2190
2195
2200
2205
2210
2215
2220
2225
2230
2235
2240
2245
2250
2255
2260
2265
2270
2275
2280
2285
2290
2295
2300
2305
2310
2315
2320
2325
2330
2335
2340
2345
2350
2355
2360
2365
2370
2375
2380
2385
2390
2395
2400
2405
2410
2415
2420
2425
2430
2435
2440
2445
2450
2455
2460
2465
2470
2475
2480
2485
2490
2495
2500
2505
2510
2515
2520
2525
2530
2535
2540
2545
2550
2555
2560
2565
2570
2575
2580
2585
2590
2595
2600
2605
2610
2615
2620
2625
2630
2635
2640
2645
2650
2655
2660
2665
2670
2675
2680
2685
2690
2695
2700
2705
2710
2715
2720
2725
2730
2735
2740
2745
2750
2755
2760
2765
2770
2775
2780
2785
2790
2795
2800
2805
2810
2815
2820
2825
2830
2835
2840
2845
2850
2855
2860
2865
2870
2875
2880
2885
2890
2895
2900
2905
2910
2915
2920
2925
2930
2935
2940
2945
2950
2955
2960
2965
2970
2975
2980
2985
2990
2995
3000
3005
3010
3015
3020
3025
3030
3035
3040
3045
3050
3055
3060
3065
3070
3075
3080
3085
3090
3095
3100
3105
3110
3115
3120
3125
3130
3135
3140
3145
3150
3155
3160
3165
3170
3175
3180
3185
3190
3195
3200
3205
3210
3215
3220
3225
3230
3235
3240
3245
3250
3255
3260
3265
3270
3275
3280
3285
3290
3295
3300
3305
3310
3315
3320
3325
3330
3335
3340
3345
3350
3355
3360
3365
3370
3375
3380
3385
3390
3395
3400
3405
3410
3415
3420
3425
3430
3435
3440
3445
3450
3455
3460
3465
3470
3475
3480
3485
3490
3495
3500
3505
3510
3515
3520
3525
3530
3535
3540
3545
3550
3555
3560
3565
3570
3575
3580
3585
3590
3595
3600
3605
3610
3615
3620
3625
3630
3635
3640
3645
3650
3655
3660
3665
3670
3675
3680
3685
3690
3695
3700
3705
3710
3715
3720
3725
3730
3735
3740
3745
3750
3755
3760
3765
3770
3775
3780
3785
3790
3795
3800
3805
3810
3815
3820
3825
3830
3835
3840
3845
3850
3855
3860
3865
3870
3875
3880
3885
3890
3895
3900
3905
3910
3915
3920
3925
3930
3935
3940
3945
3950
3955
3960
3965
3970
3975
3980
3985
3990
3995
4000
4005
4010
4015
4020
4025
4030
4035
4040
4045
4050
4055
4060
4065
4070
4075
4080
4085
4090
4095
4100
4105
4110
4115
4120
4125
4130
4135
4140
4145
4150
4155
4160
4165
4170
4175
4180
4185
4190
4195
4200
4205
4210
4215
4220
4225
4230
4235
4240
4245
4250
4255
4260
4265
4270
4275
4280
4285
4290
4295
4300
4305
4310
4315
4320
4325
4330
4335
4340
4345
4350
4355
4360
4365
4370
4375
4380
4385
4390
4395
4400
4405
4410
4415
4420
4425
4430
4435
4440
4445
4450
4455
4460
4465
4470
4475
4480
4485
4490
4495
4500
4505
4510
4515
4520
4525
4530
4535
4540
4545
4550
4555
4560
4565
4570
4575
4580
4585
4590
4595
4600
4605
4610
4615
4620
4625
4630
4635
4640
4645
4650
4655
4660
4665
4670
4675
4680
4685
4690
4695
4700
4705
4710
4715
4720
4725
4730
4735
4740
4745
4750
4755
4760
4765
4770
4775
4780
4785
4790
4795
4800
4805
4810
4815
4820
4825
4830
4835
4840
4845
4850
4855
4860
4865
4870
4875
4880
4885
4890
4895
4900
4905
4910
4915
4920
4925
4930
4935
4940
4945
4950
4955
4960
4965
4970
4975
4980
4985
4990
4995
5000
5005
5010
5015
5020
5025
5030
5035
5040
5045
5050
5055
5060
5065
5070
5075
5080
5085
5090
5095
5100
5105
5110
5115
5120
5125
5130
5135
5140
5145
5150
5155
5160
5165
5170
5175
5180
5185
5190
5195
5200
5205
5210
5215
5220
5225
5230
5235
5240
5245
5250
5255
5260
5265
5270
5275
5280
5285
5290
5295
5300
5305
5310
5315
5320
5325
5330
5335
5340
5345
5350
5355
5360
5365
5370
5375
5380
5385
5390
5395
5400
5405
5410
5415
5420
5425
5430
5435
5440
5445
5450
5455
5460
5465
5470
5475
5480
5485
5490
5495
5500
5505
5510
5515
5520
5525
5530
5535
5540
5545
5550
5555
5560
5565
5570
5575
5580
5585
5590
5595
5600
5605
5610
5615
5620
5625
5630
5635
5640
5645
5650
5655
5660
5665
5670
5675
5680
5685
5690
5695
5700
5705
5710
5715
5720
5725
5730
5735
5740
5745
5750
5755
5760
5765
5770
5775
5780
5785
5790
5795
5800
5805
5810
5815
5820
5825
5830
5835
5840
5845
5850
5855
5860
5865
5870
5875
5880
5885
5890
5895
5900
5905
5910
5915
5920
5925
5930
5935
5940
5945
5950
5955
5960
5965
5970
5975
5980
5985
5990
5995
6000
6005
6010
6015
6020
6025
6030
6035
6040
6045
6050
6055
6060
6065
6070
6075
6080
6085
6090
6095
6100
6105
6110
6115
6120
6125
6130
6135
6140
6145
6150
6155
6160
6165
6170
6175
6180
6185
6190
6195
6200
6205
6210
6215
6220
6225
6230
6235
6240
6245
6250
6255
6260
6265
6270
6275
6280
6285
6290
6295
6300
6305
6310
6315
6320
6325
6330
6335
6340
6345
6350
6355
6360
6365
6370
6375
6380
6385
6390
6395
6400
6405
6410
6415
6420
6425
6430
6435
6440
6445
6450
6455
6460
6465
6470
6475
6480
6485
6490
6495
6500
6505
6510
6515
6520
6525
6530
6535
6540
6545
6550
6555
6560
6565
6570
6575
6580
6585
6590
6595
6600
6605
6610
6615
6620
6625
6630
6635
6640
6645
6650
6655
6660
6665
6670
6675
6680
6685
6690
6695
6700
6705
6710
6715
6720
6725
6730
6735
6740
6745
6750
6755
6760
6765
6770
6775
6780
6785
6790
6795
6800
6805
6810
6815
6820
6825
6830
6835
6840
6845
6850
6855
6860
6865
6870
6875
6880
6885
6890
6895
6900
6905
6910
6915
6920
6925
6930
6935
6940
6945
6950
6955
6960
6965
6970
6975
6980
6985
6990
6995
7000
7005
7010
7015
7020
7025
7030
7035
7040
7045
7050
7055
7060
7065
7070
7075
7080
7085
7090
7095
7100
7105
7110
7115
7120
7125
7130
7135
7140
7145
7150
7155
7160
7165
7170
7175
7180
7185
7190
7195
7200
7205
7210
7215
7220
7225
7230
7235
7240
7245
7250
7255
7260
7265
7270
7275
7280
7285
7290
7295
7300
7305
7310
7315
7320
7325
7330
7335
7340
7345
7350
7355
7360
7365
7370
7375
7380
7385
7390
7395
7400
7405
7410
7415
7420
7425
7430
7435
7440
7445
7450
7455
7460
7465
7470
7475
7480
7485
7490
7495
7500
7505
7510
7515
7520
7525
7530
7535
7540
7545
7550
7555
7560
7565
7570
7575
7580
7585
7590
7595
7600
7605
7610
7615
7620
7625
7630
7635
7640
7645
7650
7655
7660
7665
7670
7675
7680
7685
7690
7695
7700
7705
7710
7715
7720
7725
7730
7735
7740
7745
7750
7755
7760
7765
7770
7775
7780
7785
7790
7795
7800
7805
7810
7815
7820
7825
7830
7835
7840
7845
7850
7855
7860
7865
7870
7875
7880
7885
7890
7895
7900
7905
7910
7915
7920
7925
7930
7935
7940
7945
7950
7955
7960
7965
7970
7975
7980
7985
7990
7995
8000
8005
8010
8015
8020
8025
8030
8035
8040
8045
8050
8055
8060
8065
8070
8075
8080
8085
8090
8095
8100
8105
8110
8115
8120
8125
8130
8135
8140
8145
8150
8155
8160
8165
8170
8175
8180
8185
8190
8195
8200
8205
8210
8215
8220
8225
8230
8235
8240
8245
8250
8255
8260
8265
8270
8275
8280
8285
8290
8295
8300
8305
8310
8315
8320
8325
8330
8335
8340
8345
8350
8355
8360
8365
8370
8375
8380
8385
8390
8395
8400
8405
8410
8415
8420
8425
8430
8435
8440
8445
8450
8455
8460
8465
8470
8475
8480
8485
8490
8495
8500
8505
8510
8515
8520
8525
8530
8535
8540
8545
8550
8555
8560
8565
8570
8575
8580
8585
8590
8595
8600
8605
8610
8615
8620
8625
8630
8635
8640
8645
8650
8655
8660
8665
8670
8675
8680
8685
8690
8695
8700
8705
8710
8715
8720
8725
8730
8735
8740
8745
8750
8755
8760
8765
8770
8775
8780
8785
8790
8795
8800
8805
8810
8815
8820
8825
8830
8835
8840
8845
8850
8855
8860
8865
8870
8875
8880
8885
8890
8895
8900
8905
8910
8915
8920
8925
8930
8935
8940
8945
8950
8955
8960
8965
8970
8975
8980
8985
8990
8995
9000
9005
9010
9015
9020
9025
9030
9035
9040
9045
9050
9055
9060
9065
9070
9075
9080
9085
9090
9095
9100
9105
9110
9115
9120
9125
9130
9135
9140
9145
9150
9155
9160
9165
9170
9175
9180
91

either with PSM-P1 (open bars), the influenza matrix protein M1 (hatched bars), or nothing (crossed bars). Standard ELISAs were performed to assess IFN γ secretion. Day 46 of culture (Fig. 6A) or Day 53 of culture (Fig. 6B).

5

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods, and compositions, for use of dendritic cells (DCs) to activate T cells for an immunotherapeutic response against an antigen. The antigen can be any antigen, including a viral or bacterial antigen, a tissue antigen, a tumor associated antigen or other antigen associated with, for example, a primary or metastatic cancer. The DCs obtained from human donors are administered to a patient to activate the relevant T cell responses *in vivo* subsequent to exposure to a virus, a bacteria or to a tissue associated, a tissue specific, a tumor or cancer associated, or tumor specific antigen in combination with a factor or agent that promotes Major Histocompatibility Complex-(MHC) class-I processing. The antigen can be a fragment of one of the antigens provided above. Further, and optionally the methods and compositions induce DC maturation. The factor or agent thus serving to promote DC activation of T cells such that at least 25%, and even over 50% of the T cells, as compared to antigen provided alone, are CD8 $^{+}$. Alternatively, the DCs are exposed to a tissue specific antigen, a cancer antigen or an antigenic fragment of either antigen with the factor promoting MHC-class I processing and maturation of DCs *in vitro* and subsequently incubated with primed or unprimed T cells to activate the relevant T cell responses *in vitro*. The activated T cells are then administered to a cancer patient in need thereof. In either case, the DCs are advantageously used to elicit an immunotherapeutic growth inhibiting response against a primary or metastatic cancer tumor.

25 Solely for ease of explanation, the description of the invention is divided into the following sections: (1) sources of antigen, (2) methods for obtaining or isolating dendritic cells, including DCs with extended lifespan or cryopreserved DCs; and (3) applications or methods of use of DCs to stimulate cytotoxic and helper T cells against a virus, bacteria or cancer *in vitro* and *in vivo*.

30 Antigen-reactive T cells are antigen-specific effector cells that are important in resisting disease, including cancer. Antigen-reactive T cells which are CD8 $^{+}$, recognize antigen presented by MHC-class I molecules. MHC-class I molecules are expressed by

almost all cell types. Antigen-reactive T cells which are CD4⁺, recognize antigen presented by MHC-class II molecules. MHC-class II molecules are expressed in a variety of cell types including dendritic cells, endothelial cells, monocytes, macrophages, and lymphocytes. The ability of antigen-reactive T cells to kill target cells is restricted by antigenic and genetic factors. For lysis of target cells, the target cells must carry the same antigen that originally induced the stimulation of the T cells, and the same class MHC molecule as the T cells.

The present invention relates to methods of generating T cells reactive to an antigen that can be used in the prevention or treatment of a disease or disorder, such as a viral or bacterial infection, or cancer. This invention was made possible by the surprising discovery that bacillus Calmette Guerin (BCG) stimulates MHC-class I processing of exogenous soluble antigen and subsequently increases preferential activation of CD8⁺ T cells to at least 25%, and even greater than 50% of the activated T cell population, when compared to individuals administered antigen alone. The proportion of CD8⁺ T cells can increase to 25%, 50%, or more, and can be even greater than 75% of the total T cell response.

As an alternative embodiment, BCG with a viral, a bacterial, a tissue associated, tissue specific, tumor associated or tumor specific antigen, or an antigenic fragment thereof can be added multiple times to the *in vitro* cultures in order to restimulate antigen-reactive T-cell proliferation. The antigen-reactive T cells generated by the methods of the invention are capable of specifically targeting, killing, or causing lysis of the infected cells or cancer cells, or other target cells as the case may be, or any cells bearing the same antigens and similar MHC molecules with which the T cells are prepared. The antigen-reactive T cells of the invention can also secrete one or more measurable cytokines, such as IL-2, IFN- γ , TNF- β , IL-4, IL-5, IL-6, IL-9, IL-10, IL-3, and/or GM-CSF. The production of these cytokines can be used to monitor specific T cell activation *in vitro*.

Previously, BCG has been used as a constituent of various vaccine compositions to act as an adjuvant for augmenting a serological or antibody immune response to the target immunogen. Further, dendritic cells have been shown to internalize particles, including BCG mycobacteria. (Inaba, et al., *J. Exp. Med.* 178:479-488 (1993)). The mycobacteria-laden dendritic cells have been shown to be more potent in presenting antigens to primed T cells then corresponding cultures of mature dendritic cells that are exposed to a pulse of BCG. (Inaba et al., *supra* (1993)).

Dendritic cell activation by BCG has been characterized as involving homotypic aggregation, up regulation of surface antigens, down modulation of endocytic activity and the release of tumor necrosis factor- α . (Thurnher, et al., *Int. J. Cancer* 70:128-134 (1997)). Enhanced expression has been documented for dendritic-cell-maturation antigen CD83 and of the T cell co-stimulator CD86 (B7-2). It has been suggested that induction of secretion of TNF- α was at least in part responsible for the observed phenotypic and functional changes observed in dendritic cells following uptake of BCG. Stimulation of IL-8 mRNA expression and IL-8 protein secretion has also been associated with T cell effects of BCG. (Ramoner et al., *J. Urology* 159:1488-1492 (1998)).

To date, although BCG has been administered in conjunction with various antigens, including cancer cells and cancer associated antigens, there has apparently been no demonstration or recognition of a preferential activation of CD8 $^{+}$ T cells when exposed to dendritic cells that have been activated with tissue specific antigen combined with BCG, or with BCG and LPS inducing a MHC-class I response.

The antigen-reactive T cells can be administered *in vivo* autologously (*i.e.*, to the same individual from which the T cells (or parental cells to the T cells) were originally obtained) or syngeneically (*i.e.*, to an identical twin of the individual from which the cancer or infected cells were initially obtained); or allogeneically to an individual who shares at least one common MHC allele with the individual from which the antigenic cells and T cells were originally obtained.

As used herein, the term “antigenic cells” refers to any cells, typically infected cells or cancer cells, and in particular, prostate cancer cells, which can elicit an immune response in a subject. The sources of antigenic cells, and methods of preparation of antigenic cells for use in the present methods are discussed in this section.

The term “pulsed” as used herein includes the process of immunization *in vitro*. The process of immunization *in vitro* can be performed by a variety of methods including but not limited to the dendritic cells pulsed with antigens (Steel and Nutman, *J. Immunol.* 160:351-360 (1998); Tao et al., *J. Immunol.* 158:4237-4244 (1997); Dozmorov and Miller, *Cell Immunol.* 178:187-196 (1997); Inaba et al., *J Exp Med.* 166:182-194 (1987); Macatonia et al., *J Exp Med.* 169:1255-1264 (1989); De Bruijn et al., *Eur. J. Immunol.* 22:3013-3020 (1992)), RMA-S cells (mutant cells expressing high numbers of ‘empty’ cell surface class I MHC molecules) loaded with peptide (De Bruijn et al., *Eur. J. Immunol.*

21:2963-2970 (1991); De Bruijn et al., *Eur. J. Immunol.* 22:3013-3020 (1992); Houbiers et al., *Eur. J. Immunol.* 26:2072-2077 (1993)) and macrophage phagocytosed-peptide loaded beads (De Bruijn et al., *Eur. J. Immunol.* 25:1274-1285 (1995)), and osmotically stressed antigenic cells (PCT publication WO 98/15616)). Priming, therefore, results in the first

5 exposure of naive immune cells to an antigenic molecule.

The term “pulsing” as used herein refers to the process of exposing primed immune cells *in vitro* to BCG, and alternatively BCG and LPS, and a viral antigen, bacterial antigen, tissue specific antigen, a tumor antigen, or an antigenic fragment of the antigen. The BCG and viral antigen, bacterial antigen, tissue specific or tumor associated antigens, as used

10 herein, comprises a non-covalent mixture of BCG and an antigenic molecule.

The term “antigen,” and "antigenic molecule" as used herein comprises viral, bacterial, tissue associated or tissue specific and tumor associated or tumor specific protein antigens useful for presentation by the dendritic cells to activate T cells for immunotherapeutics. In particular, for developing an immune response to the infecting virus or bacteria or to prostate cells or prostate associated antigens, i.e., PSMA in prostatic tumor vasculature.

According to one embodiment, a prostate tumor cell lysate recovered from surgical specimens can be used as antigen. For example, a sample of a cancer patient's own tumor, obtained at biopsy or at surgical resection, can be used to provide a cell lysate for antigen. Alternatively, a membrane preparation of tumor cells of a prostate cancer patient can be used.

According to yet another embodiment, purified prostate specific membrane antigen (PSMA, also known as PSM antigen), which specifically reacts with monoclonal antibody 7E11-C.5 (*see generally* Horoszewicz et al., *Prog. Clin. Biol. Res.* 37:115-132 25 (1983), US Patent No. 5,162,504, US Patent No. 5,788,963, Feng et al., *Proc. Am. Assoc. Cancer Res.* 32:(Abs. 1418)238 (1991)) can be used as antigen. Cloning of the gene encoding the PSMA antigen has been described by Israeli et al., *Cancer Res.* 54:1807-1811. Expression of the cloned gene, *e.g.*, in yeast cells, can provide a ready source of the PSMA antigen for use according to the present invention.

In still yet another embodiment, an antigenic peptide having the amino acid residue sequence Leu Leu His Glu Thr Asp Ser Ala Val (SEQ ID NO: 1)(designated PSM-P1) which corresponds to amino acid residues 4-12 of PSMA can be used as antigen.

According to another embodiment, an antigenic peptide having the amino acid residue sequence Ala Leu Phe Asp Ile Glu Ser Lys Val (SEQ ID NO: 2) (designated PSM-P2) which corresponds to amino acid residues 711-719 of PSMA can be used as antigen.

According to another embodiment, an antigenic peptide having an amino acid residue sequence Xaa Leu (or Met) Xaa Xaa Xaa Xaa Xaa Xaa Val (or Leu) (designated PSM-PX) where Xaa represents any amino acid residue can be used as antigen. This peptide resembles the HLA-A0201 binding motif, *i.e.*, a binding motif of 9-10 amino acid residues with "anchor residues", leucine (Leu) and valine (Val) found in HLA-A2 patients (Grey et al., *Cancer Surveys* 22:37-49 (1995)). This peptide is typically used as antigen for HLA-A2⁺ patients. (see, *Central Data Analysis Committee "Allele Frequencies"*, Section 6.3, Tsuji, K. et al., (eds.), Tokyo University Press, pp. 1066-1077).

In yet another embodiment, an antigenic peptide selected from the peptides listed in Table 1A (below) can be used as antigen. The peptides listed in Table 1A have amino acid residue sequences corresponding to fragments of PSM and have been matched to a binding motif of a specific haplotype. According to one embodiment, the peptides are selected to be presented by dendritic cells to activate T cells of a patient which matched the haplotype indicated for each peptide in Table 1A.

Table 1A
PSM Peptides*

ID No.	Haplotype	Initial Amino Acid Residue**	Amino Acid Sequence	SEQ ID NO:
PSM-P3	A2	20	Trp Leu Cys Ala Gly Ala Leu Val Leu	3
PSM-P4	A2	27	Val Leu Ala Gly Gly Phe Phe Leu Leu	4
PSM-P5	A2	109	Glu Leu Ala His Tyr Asp Val Leu Leu	5
PSM-P6	A2	260	Asn Leu Asn Gly Ala Gly Asp Pro Leu	6
PSM-P7	A2	461	Thr Leu Arg Val Asp Cys Thr Pro Leu	7
PSM-P8	A2	660	Val Leu Arg Met Met Asn Asp Gln Leu	8
PSM-P9	A2	568	Pro Met Phe Lys Tyr His Leu Thr Val	9
PSM-P10	A2	57	Asn Met Lys Ala Phe Leu Asp Glu Leu	10
PSM-P11	A2	469	Leu Met Tyr Ser Leu Val His Asn Leu	11
PSM-P12	A2	663	Met Met Asn Asp Gln Leu Met Phe Leu	12
PSM-P16	A1	171	Glu Gly Asp Leu Val Tyr Val Asn Tyr	13
PSM-P17	A1	264	Ala Gly Asp Pro Leu Thr Pro Gly Tyr	14
PSM-P18	A1	463	Arg Val Asp Cys Thr Pro Leu Met Tyr	15
PSM-P19	A1	143	Leu Phe Glu Pro Pro Pro Gly Tyr	16
PSM-P20	A1	558	Thr Tyr Glu Leu Val Glu Lys Phe Tyr	17
PSM-P21	A1	701	Ala Gly Glu Ser Phe Pro Gly Ile Tyr	18
PSM-P22	A1	725	Trp Gly Glu Val Lys Arg Gln Ile Tyr	19
PSM-P26	A11	398	Ile Val Arg Ser Phe Gly Thr Leu Lys Lys Glu	20
PSM-P27	A11	63	Asp Glu Leu Lys Ala Glu Asn Ile Lys Lys Phe	21
PSM-P28	A11	491	Lys Ser Leu Tyr Glu Ser Trp Thr Lys Lys Ser	22
PSM-P36	A24	448	Ala Tyr Ile Asn Ala Asp Ser Ser Ile	23
PSM-P37	A24	606	Lys Tyr Ala Asp Lys Ile Tyr Ser Ile	24
PSM-P38	A24	298	Gly Tyr Tyr Asp Ala Gln Lys Leu Leu	25
PSM-P39	A24	624	Thr Tyr Ser Val Ser Phe Asp Ser Leu	26
PSM-P40	A24	178	Asn Tyr Ala Arg Thr Glu Asp Phe Phe	27
PSM-P41	A24	227	Leu Tyr Ser Asp Pro Ala Asp Tyr Phe	28
PSM-P46	B3501	289	Leu Pro Ser Ile Pro Val His Pro Ile	29
PSM-P47	B3501	501	Ser Pro Ser Pro Glu Phe Ser Gly Met	30

* "PSM peptides" refers to peptides having an amino acid sequence corresponding to a fragment of PSMA (a/k/a PSM).

** "Initial Amino Acid Residue" refers to the residue number of the amino acid of PSM to which the first amino acid of the peptide corresponds.

In another embodiment of the present invention, prostate specific antigen (PSA) (see, Pepsidero et al., *Cancer Res.* 40:2428-2432 (1980); McCormack et al., *Urology* 45:729-744 (1995)) can be used as antigen.

5 According to another embodiment, an antigenic peptide selected from the peptides listed in Table 1B (below) can be used as antigen. The peptides in Table 1B have amino acid residue sequences corresponding to fragments of PSA and have been matched to a binding motif of a specific haplotype as indicated in Table 1B. According to one embodiment, the peptide is presented by dendritic cells to activate T cells of patients which 10 match the haplotype indicated for each peptide in Table 1B.

Table 1B
PSA Peptides*

Iden. No.	Haplotype	Initial Amino Acid Residue**	Amino Acid Sequence	SEQ ID NO.
PSA-P1	A2	53	Val Leu Val His Pro Gln Trp Val Leu	31
PSA-P2	A2	171	Lys Leu Gln Cys Val Asp Leu His Val	32
PSA-P11	A1	235	Ala Leu Pro Glu Arg Pro Ser Leu Tyr	33
PSA-P21	A11	25	Ile Val Gly Gly Trp Glu Cys Glu Lys	34
PSA-P22	A11	185	Gln Val His Pro Gln Lys Val Thr Lys	35
PSA-P23	A11	245	Val Val His Tyr Arg Lys Trp Ile Lys	36
PSA-P31	A24	152	Cys Tyr Ala Ser Gly Trp Gly Ser Ile	37

* "PSA peptides" refers to peptides having an amino acid sequence corresponding to a fragment of PSA.

15 ** "Initial Amino Acid Residue" refers to the residue number of the amino acid of PSA to which the first amino of the peptide corresponds.

According to still another embodiment, a prostate mucin antigen, recognized by monoclonal antibody PD41, described by Wright (US Patent No. 5,227,471 and No. 5,314,996; Beckett et al. *Cancer Res.* 51:1326-1222 (1991)) can be used as antigen.

Alternatively, a crude lysate of prostate tumor cells comprising antigen which binds to the 5 antibody produced by the hybridoma cell line ATCC HB 11094 and which express the PD41 mucin antigen can be used as antigen.

Additional prostate antigens which can be used in the methods of the present invention include, but are not limited to, six transmembrane epithelial antigen of the prostate (STEAP; Hubert et al., *Proc. Natl. Acad. Sci. USA* 96:14523-14528 (1999)), prostate carcinoma tumor antigen (PCTA-1; Su et al. *Proc. Natl. Acad. Sci. USA* 93:7252-7257 10 (1996)); prostate stem cell antigen (PSCA; Reiter, et al., *Proc. Natl. Acad. Sci. USA* 95:1735-1740 (1998)). Antigenic fragments of each antigen are also considered to be encompassed by the scope of the present invention.

Additional antigens include, but are not limited to, viral neutralization antigens or antigenic peptides. Further, bacterial proteins, glycoproteins, glycolipids or carbohydrates and antigenic fragments thereof, are considered part of the present invention.

According to the present invention, cellular immunotherapy is developed by obtaining antigenic cells and immune cells from one or more individuals, more typically from the same subject, and stimulating T cells within the immune cell population by the methods of the invention. This *in vitro* stimulation of T cells, followed by clonal expansion in cell culture of antigen-reactive CD4⁺ and/or CD8⁺ T cells, and administration of the antigen-reactive T cells into the subject, constitute a useful therapeutic and prophylactic strategy. When infused into the subject, antigen-reactive T cells of the invention can specifically target and/or directly kill target cells *in vivo* that bear the same antigen as the antigenic cells, thereby inhibiting cancer development and/or tumor cell proliferation, or preventing or 25 limiting the spread of a pathogen in the recipient.

In one embodiment of the invention, the antigenic cells, the T cells to be activated, and the recipient of the antigen-reactive T cells have the same MHC (HLA) haplotype. In another embodiment, the invention is directed to the use of autologous T cells 30 stimulated *in vitro* with autologously-derived antigen for the treatment of cancer, inhibition of tumor cell proliferation, or prevention of cancer development in the same subject from which the T cells (or more typically, all the immune cells) and antigen were originally

derived. In one particular aspect, the immune cells and antigenic cells are isolated from a human subject in need of cellular immunotherapy.

In another embodiment of the invention, the T cells and the recipient have the same haplotype while the antigenic cells are allogeneic to the T cells and the recipient, but matched at least one MHC allele, i.e., antigenic cells are used to activate T cells, which T cells are then administered to a recipient from which the T cells were originally obtained, and in which the antigenic cells and the T cells share at least one but not all MHC alleles. In a less typical embodiment of the invention, the antigenic cells, the T cells and the recipient are all allogeneic with respect to each other, but all have at least one common MHC allele shared among the antigenic cells, the T cells and the recipient.

According to the invention, the methods for generating antigen-reactive T lymphocytes comprise priming live immune cells, pulsing the primed immune cells with BCG and a tissue associated, tissue specific, tumor associated, or tumor specific antigen (with or without LPS), whereas the immune cells comprise APCs, for example, but not limited to dendritic cells, and co-cultured, pulsed cells with primed T cells. In one embodiment, the primed immune cells are enriched for APCs prior to pulsing. In another embodiment, the primed immune cells are separated to generate enriched or purified populations of T cells or APCs. In a specific embodiment, primed immune cells are separated to generate enriched or purified populations of CD4⁺ T cells prior to pulsing. Co-culturing of pulsed cells with T cells lead to the stimulation of specific T cells which mature into antigen-reactive CD4⁺ T cells or antigen-reactive CD8⁺ T cells respectively.

Without limitation of the present invention to any particular scientific model or mechanism, the results described herein suggest that BCG with pulsed immune cells (those cells pulsed with a viral antigen, a bacterial antigen, a tissue associated, or tissue specific antigen, a tumor associated or tumor specific antigen, or antigenic fragments thereof as set forth above) comprising APCs are uniquely enabled to induce a specific activation of CD8⁺ T cells *in vitro* directed against virus, bacterial, infected or tumor cells. The results described herein further suggest that a maturation promoting factor can be added to enhance the duration of the immune response. BCG serves to increase DC expression of the surface maturation markers CD83 and CD86, concomitant with exclusion of antigens from endocytosis. Furthermore, lipopolysaccharide (LPS) also down-regulates endocytic activity and promotes DC maturation, potentially increasing the duration of the immune response.

In another embodiment of the invention, the methods can further comprise restimulation of the antigen reactive T cells *in vitro*, by culturing the cells with feeder cells and irradiated antigenic cells, optionally in the presence of a composition comprising one or more cytokines (*e.g.*, purified IL-2, Concanavalin A-stimulated spleen cell supernatant). *In vitro* restimulation of T cells by addition of APCs and BCG with soluble exogenous antigen, *i.e.*, a viral, a bacterial, a tumor associated antigen or tissue specific antigen or an antigenic fragment of either antigen to the culture can be used to promote expansion of the T cell populations.

In another embodiment, the T cells are stimulated with irradiated spleen cells or APCs purified from peripheral blood as feeder cells in the presence of BCG and viral, bacterial, tissue specific antigen or tumor antigen or an antigenic fragment of either antigen (with or without LPS). In this manner, by restimulation from time-to-time, a stable antigen-specific T cell culture or cell line can be maintained *in vitro* for long periods of time. The T cell culture or cell line thus created can be stored, and if preserved (*e.g.*, by formulation with a cryopreservative and freezing) used to resupply antigen-reactive T cells at desired intervals for long term use.

According to a specific embodiment of the invention, antigen-reactive CD8⁺ T cells can be generated and used prophylactically to prevent the progression (proliferation of virus, bacteria or tumor cells) or development of a tumor, or to induce remission of cancer. Antigen-reactive CD4⁺ T cells can also be generated and used prophylactically to prevent the progression or development of a tumor (proliferation of tumor cells) or to induce remission of cancer. In another embodiment, the T cells can be used therapeutically to treat cancer. Typically, the antigenic cells used to generate the antigen-reactive T cells are syngeneic to the subject to which they are to be administered, *e.g.*, are obtained from the subject. However, if antigenic cells that are syngeneic to the subject are not available for use, the methods of the invention provide that such antigenic cells having the same HLA haplotype as the intended recipient of the cells can be prepared *in vitro* using noncancerous cells (*e.g.*, normal cells) collected from the recipient. In still another embodiment, lysates or preparations of tumor cells, can be used for restimulation of antigen-reactive T cells of the invention.

In another embodiment, normal cells can be induced to become cancerous or transformed, *e.g.*, by treatment with carcinogens, such as chemicals and/or radiation or

infection with a transforming virus, and then used for pulsing directly or used to prepare a lysate for pulsing dendritic cells in combination with BCG or BCG combined with LPS.

In another embodiment, lysates or preparations of such tissue associated or tissue specific; cancerous or transformed cells, and the like, can be used to pulse immune 5 cells or APCs *in vitro*. In still another embodiment, the lysates or preparations of such cells, can be used for restimulation of the antigen-reactive T cells of the invention.

Furthermore, in another embodiment, if the cloned gene of an antigen of interest is available, normal cells from the subject can be transformed or transfected with the 10 gene, such that the antigen of interest is expressed recombinantly in the cells, and then such cells can be used in the priming, pulsing, and/or restimulation reactions. In a less typical aspect, antigenic cells for use can be prepared from cells that are not syngeneic but that have 15 at least one MHC allele in common with the intended recipient.

In an immune response, the process of antigen-induced T cell activation occurs *in vivo* typically in secondary lymphoid tissues, such as the lymph nodes and the 20 spleen. By following the present methods, any antigenic cell of interest can be used to prime T cells *in vitro*, even cancer cells or infected cells that are considered unsafe for use in active immunization. Such primed T cells are then exposed to APCs pulsed with viral, bacterial, tissue specific antigen, tumor antigen, or antigenic fragments of either antigen and BCG. In a specific embodiment CD8⁺ antigen-reactive T cells are expanded *in vitro* as a source of cells for immunotherapy. Thus, one advantage of the present methods is that antigen-specific T 25 cells can be expanded *in vitro* to create a source of cells for immunotherapy that can be used for treatment or prevention of disease.

There are many advantages of immunotherapy as provided by the present invention. Tumor bulk is minimal following surgery and immunotherapy is most effective in 30 this situation. In a specific embodiment, the methods of the invention are directed at enhancing the immunocompetence of a cancer patient either before surgery or after surgery, and enhancing cell-mediated tumor-specific immunity against cancer cells, with the objective being inhibition of proliferation of cancer cells, and total eradication of residual cancer cells in the body. In another aspect, antigen-reactive T cells reactive against human cancer cells can be used, alone or in conjunction with surgery, chemotherapy, radiation or other anti-cancer therapies, to eradicate metastases or micrometastases, or to purge bone marrow of cancer cells during bone marrow transplantation. For example, to eradicate or inhibit the

growth of metastases or micrometastases, the antigen-reactive T cells provided by the invention, typically CD3⁺CD8⁺ or CD3⁺CD4⁺ T cells are administered *in vivo*, to the subject having or suspected of having a metastases or micrometastases.

- As an illustration, to purge bone marrow of cancer cells during bone marrow transplantation, bone marrow from the donor is contacted *in vitro* with the antigen-reactive T cells provided by the invention, so that the antigen reactive T cells lyse any residual cancer cells in the bone marrow, prior to administering the bone marrow to the subject for purposes of hematopoietic reconstitution. The bone marrow transplantation is typically autologous. In one embodiment, the antigen-reactive T cells are CD3⁺CD8⁺ or CD3⁺CD4⁺ T cells.
- 10 Alternatively, administration of the antigen-reactive T cells involves both CD4⁺ T cells and CD8⁺ T cells.

Moreover, if cancer patients undergo surgery with anesthesia, and subsequent chemotherapy, the resulting immunosuppression experienced by the patient can be lessened by cellular immunotherapy in the preoperative period, thereby reducing the incidence of infectious complications. There is also the possibility that tumor cells are shed into the circulation at surgery, and thus, effective immunotherapy applied at this time can eliminate these cells *in vivo*. The invention thus provides a method of prophylaxis or treatment comprising administering to a cancer patient the antigen-reactive T cells provided by the present invention, reactive against an antigen of the patient's cancer cells, prior to, during, and/or subsequent to surgery and/or chemotherapy undergone by the cancer patient.

A number of antigens or antigenic compositions are useful, according to the present invention, for presentation by the DCs to activate T cells for immunotherapeutics. In one embodiment, a prostate cancer tumor cell lysate recovered from surgical specimens is used as an antigen. For example, a sample of a cancer patient's own tumor, obtained at biopsy or at surgical resection, can be used to provide a cell lysate for antigen. Alternatively, a membrane preparation of tumor cells of a cancer patient, e.g., a prostate cancer patient, or established cell lines can be used as an antigen. Additional antigens useful in the present methods including viral and bacterial antigens, are discussed in detail above.

According to the present invention, DCs can be exposed to a desired viral, bacterial, tissue associated or tissue specific antigen, prostate cancer associated antigen, or an antigenic fragment of the antigens by incubating the DCs with the antigen in *in vitro* culture medium. In one embodiment, the antigen, in aqueous soluble or aqueous suspension form in

combination with BCG alone or in combination with BCG and LPS, are added to cell culture medium. As demonstrated herein, the DCs advantageously take up antigen for successful presentation to T cells in the context of MHC-class I.

In another embodiment, antigens are introduced to the cytosol of the DCs by alternate methods, including, but not limited, to osmotic lysis of pinocytic vesicles and the use of pH sensitive liposomes, and the like. See, generally, (Okada et al., *Cell* 29:33 (1982); Poste et al., *Methods Cell Biol.* 14:33 (1976); Reddy et al., *J. Immunol. Methods* 141:157 (1991)).

10 Isolation of Dendritic Cells

Human dendritic cells (DCs) are obtained from any tissue where they reside including non-lymphoid tissues such as the epidermis of the skin (Langerhans cells) and lymphoid tissues such as the spleen, bone marrow, lymph nodes and thymus. DCs can also be isolated as well from the circulatory system including blood (blood DCs) and lymph (veiled cells). Human peripheral blood is an easily accessible ready source of human DCs and is used as a source according to a specific embodiment of the invention. Cord blood is another source of human DCs and in cases where a child is born into a family known to be at high risk for prostate cancer, cord blood can be used as a source of DCs which can be cryopreserved for later use, if needed.

Because DCs occur in low numbers in any tissue in which they reside, including human peripheral blood, DCs must be enriched or isolated for use. Any of a number of procedures entailing repetitive density gradient separation, positive selection, negative selection or a combination thereof can be used to obtain enriched populations or isolated DCs. Examples of such methods for isolating DCs from human peripheral blood include: (O'Doherty et al., *J. Exp. Med.* 178:1067-1078 (1993); Young and Steinman, *J. Exp. Med.* 171:1315-1332 (1990); Freudenthal and Steinman, *Proc. Natl. Acad. Sci. USA* 87:7698-7702 (1990); Macatonia et al., *Immunol.* 67:285-289 (1989) and Markowicz and Engleman, *J. Clin. Invest.* 85:955-961 (1990)). A method for isolating DCs from human peripheral blood which avoids exposure of the cells to sheep red blood cells and/or fetal calf serum is described in PCT Publication W094/02156. An example of a method for isolating DCs from lymphoid tissue is described in (Macatonia et al., *J. Exp. Med.* 169:1255-1264 (1989)).

Once the DCs are obtained, they are cultured in appropriate culture medium to expand the cell population and/or to maintain the DCs in a state for optimal antigen uptake, processing and presentation.

According to one embodiment, which also provides maintenance of the proper state of "maturity" of DCs in *in vitro* culture, is to culture the DCs the presence of both granulocyte/macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4). In one example, a combination of GM-CSF and IL-4 at a concentration of about 500 units/ml of each. A recent study reveals optimal antigen presentation by "immature" vs. mature DCs (Koch et al., *J. Immunol.* 155:93-100 (1995)). Immature DCs may be used according to certain embodiments of the present invention. Recent experiments have shown that mature pulsed DCs retain the ability to stimulate a T cell response up to ten times longer than immature pulsed DCs.

As illustrated in the examples, *infra*, human DCs were isolated from peripheral blood of prostate cancer patients, and after about 5 days in *in vitro* culture, DCs competent and able to activate prostate cancer specific T cells were recovered. According to one embodiment of the invention, DCs are obtained from a cancer patient to be treated. The DCs are pulsed with one of the various antigens provided herein in the presence of BCG with or without LPS, and then used to activate autologous T cells of the patient, either *in vitro* or *in vivo*, for cancer immunotherapy and/or tumor growth inhibition.

According to an alternative embodiment, DCs are obtained from a healthy individual known not to be suffering from cancer. The relevant HLA antigens (both MHC class I and II, e.g., HLA-A, B, C and DR) on the individual's peripheral blood mononuclear cells (PBMCs) are identified and DCs which provide an HLA match with the cancer patient are isolated and expanded as described above. For example, in certain instances, late stage prostate cancer patients who have been treated with radiation and/or chemotherapy agents often are not able to provide sufficient or efficient DCs. Thus, DCs from healthy HLA-matched individuals, such as siblings, can be obtained and expanded using any of the methods described above and incubated *in vitro* with a relevant antigen in the presence of BCG to form activated natural DCs which in turn can be used to elicit activated T cells for immunotherapy and/or to inhibit tumor growth in the HLA-matched prostate cancer patient.

According to another embodiment of the invention, "extended life span dendritic cells" are used. Human cells have a finite life span *in vitro* usually limited to

approximately 50-70 population doublings before undergoing apoptosis. As used herein, the term "extended life span dendritic cells" is intended to mean DCs that have been genetically modified so that they can be expanded in *in vitro* cell culture medium for an extended period of time, including but not limited to at least about 100 additional population doublings.

- 5 Extended life span DCs are obtained, for example, by EBV-transformation of DCs obtained from peripheral blood of prostate cancer patients, or by insertion into DCs, using techniques known to those skilled in the art, of a specific cell cycle regulatory gene including but not limited to a gene which encodes cyclin A, B, D or E, or retinoblastoma protein.

As illustrated in the examples presented in U.S. Patent No. 5,788,963 extended
10 life span DCs have been obtained by EBV transformation of a population of isolated DCs. Such extended life span DCs are useful according in the methods of the present invention.

According to another embodiment of the invention, DCs can be preserved, e.g., by cryopreservation either before exposure or following exposure to a relevant antigen. Cryopreservation agents which can be used include but are not limited to dimethyl sulfoxide (DMSO) (Lovelock and Bishop, *Nature* 183:1394-1395 (1959); Ashwood-Smith, *Nature* 190:1204-1205 (1961)), glycerol, polyvinylpyrrolidone (Rinfret, *N.Y. Acad. Sci.* 85:576 (1960)), polyethylene glycol (Sloviter and Ravdin, *Nature* 196:548 (1962)), albumin, dextran, sucrose, ethylene glycol, i-erythritol, D-ribitol, D-mannitol (Rowe et al., *Fed. Proc.* 21:157- (1962)), D-sorbitol, i-inositol, D-lactose, choline chloride (Bender et al., *J. Appl. Physiol.* 15:520 (1960), amino acids (Phan and Bender, *Exp. Cell Res.* 20:651 (1960)), methanol, acetamide, glycerol monoacetate (Lovelock, *Biochem. J.* 56:265 (1954)), and inorganic salts (Phan and Bender, *Proc. Soc. Exp. Biol. Med.* 104:388 (1960); Phan and Bender, *in Radiobiology, Proceedings of the Third Australian Conference on Radiobiology*, Ilbery, P.L.T., ed., Butterworth, London, p. 59 (1961)).

25 A controlled slow cooling rate is critical. Different cryoprotective agents (Rapatz et al., *Cryobiology* 5:18-25 (1968)) and different cell types have different optimal cooling rates (see, e.g., Rowe and Rinfret, *Blood* 20:636 (1962); Rowe, *Cryobiology* 3:12-18 (1966); Lewis et al., *Transfusion* 7:17-32 (1967); and Mazur, *Science* 168:939-949 (1970)), for effects of cooling velocity on survival of marrow-stem cells and on their transplantation potential). The heat of fusion phase where water turns to ice should be minimal. The cooling procedure can be carried out by use of, e.g., a programmable freezing device or a methanol bath procedure. Programmable freezing apparatuses allow determination of optimal cooling

rates and facilitate standard reproducible cooling. Programmable controlled-rate freezers such as Cryomed or Planar permit tuning of the freezing regimen to the desired cooling rate curve.

After thorough freezing, cells can be rapidly transferred to a long-term
5 cryogenic storage vessel. In a particular embodiment, samples can be cryogenically stored in liquid nitrogen (-196°C) or its vapor (-165°C). Such storage is greatly facilitated by the availability of highly efficient liquid nitrogen refrigerators.

Considerations and procedures for the manipulation, cryopreservation, and long term storage of hematopoietic stem cells, particularly from bone marrow or peripheral
10 blood, is largely applicable to the DCs prepared by the methods of the invention. Such a discussion can be found, for example, in the following references, incorporated by reference herein: (Gorin, *Clinics in Haematology* 15:19-48 (1986); Bone-Marrow Conservation, Culture and Transplantation, Proceedings of a Panel, Moscow, July 22-26, 1968, International Atomic Energy Agency, Vienna, pp. 107-186)). Other methods of
15 cryopreservation of viable cells, or modifications thereof, are available and envisioned for use (e.g., cold metal-mirror techniques; Livesey and Linner, *Nature* 327:255 (1987); Linner et al., *J. Histochem. Cytochem.* 34:1123-1135 (1986); see also U.S. Patent No. 4,199,022 to Senken et al., U.S. Patent No. 3,753,357 to Schwartz, U.S. Patent No. 4,559,298 to Fahy, and U.S.
20 Patent No. 5,364,756 to Livesey, et al.

Frozen cells are typically thawed quickly (e.g., in a water bath maintained at 37-41°C) and chilled immediately upon thawing. It may be desirable to treat the cells in order to prevent cellular clumping upon thawing. To prevent clumping, various procedures can be used, including but not limited to, the addition before and/or after freezing of DNase (Spitzer et al., *Cancer* 45:3075-3085 (1980)), low molecular weight dextran and citrate,
25 hydroxyethyl starch (Stiff et al., *Cryobiology* 20:17-24 (1983)), and the like. The cryoprotective agent, if toxic in humans, should be removed prior to administration of the thawed DCs to an individual. One way in which to remove the cryoprotective agent is by dilution to an insignificant concentration. Once frozen DCs have been thawed and recovered, they are used to activate T cells as described above with respect to non-frozen DCs.

Applications or Methods of Use

Solely for the ease of presentation, the following discussion is illustrative of particular uses of the methods of the invention as would be clearly understood by those with skill in the relevant art. The examples provided herein are not meant to limit the scope of the present invention which can be used for essentially any antigen, including virus and bacteria for all cancer types.

As discussed *supra*, according to one embodiment of the invention, isolated human DCs, exposed to a soluble exogenous prostate specific antigen and BCG by any of the methods disclosed herein can be used to activate T cells *in vitro* against prostate cancer. In particular, the T cell response is a MHC class I-directed response providing a population of activated T cells comprising greater than 25% CD8⁺ T cells. The DCs can be used immediately after exposure to antigen to stimulate T cells. Alternatively, the DCs can be maintained in the presence of a combination of GM-CSF and IL-4 prior to simultaneous exposure to antigen and T cells.

T cells or a subset of T cells can be obtained from various lymphoid tissues for use as responder cells. Such tissues include but are not limited to spleen, lymph nodes, and peripheral and cord blood. The cells can be co-cultured with DCs exposed to antigen as a mixed T cell population or as a purified T cell subset. For example, it may be desired to culture purified CD8⁺ T cells with antigen exposed DCs to elicit prostate specific cytotoxic T lymphocytes (CTLs). In addition, early elimination of CD4⁺ T cells during *in vitro* cell culture can prevent the overgrowth of CD4⁺ cells in a mixed culture of both CD8⁺ and CD4⁺ T cells. T cell purification can be achieved by positive, and/or negative selection, including but not limited to, the use of antibodies directed to CD2, CD3, CD4, CD8, and the like.

According to one embodiment, the T cells are obtained from the same prostate cancer patient from which the DCs were obtained. After stimulation or activation *in vitro*, the autologous T cells are administered to the patient to provoke and/or augment an existing immune response which slows or inhibits prostate cancer tumor growth. For example, T cells can be administered, by intravenous infusion, at doses of about 10⁸-10⁹ cells/m² of body surface area (see, Ridell et al., *Science* 257:238-241 (1992)). Infusion can be repeated at desired intervals, for example, monthly. Recipients are monitored during and after T cell infusions for any evidence of adverse effects.

According to another embodiment, the T cells are obtained from a prostate cancer patient and the DCs which are used to stimulate the T cells are obtained from an HLA-matched healthy donor. According to yet another embodiment, both the T cells and the DCs are obtained from an HLA-matched healthy donor, e.g., a sibling of the prostate cancer patient. This embodiment can be advantageous, for example, when the patient is a late stage prostate cancer patient who has been treated with radiation and/or chemotherapy agents and may not be able to provide sufficient or efficient DCs. The T cells after stimulation, are administered as described above.

In a specific example of the methods of the present invention, PSMA loaded into dendritic cells (DCs) using various methodologies produced antigen-presenting cells that can stimulate autologous and allogeneic T cells in an antigen-specific manner. These methodologies include: 1) overnight treatment of about day 6 DCs with PSMA protein and bacillus Calmette-Guérin mycobacteria (BCG) with or without lipopolysaccharide (LPS), and 2) osmotic loading of about day 7 DCs using hypertonic medium. BCG stimulated DCs demonstrate elevated CD83 and CD86 expression while LPS further enhances DC maturation. Osmotic loading was accomplished using hypertonic medium to increase phagocytosis and macropinocytosis.

Following two weekly stimulations *in vitro* with PSMA-loaded DCs (prepared using BCG ± LPS or osmotic loading) and one or more weekly restimulations with PBMC exogenously pulsed with PSMA, specific reactivity to the immunogen was demonstrated (Fig. 1-6).

According to another embodiment of the invention, DCs isolated from a prostate cancer patient are cultured *in vitro*, then exposed to a prostate tissue specific antigen, a prostate cancer antigen, or an antigenic fragment of either antigen in a manner sufficient to obtain MHC-class I antigen presentation and which increases the relative number of CD8⁺ CTLs. After either expansion or cryopreservation, DCs are administered back to the patient to stimulate an immune response, including T cell activation, against the patient's cancer cells *in vivo*. Using this approach with the patient's own dendritic cells provides the following advantages: (1) no foreign DNA is utilized; (2) infection of cells for purposes of cDNA expression using various viral vectors are eliminated; (3) antigen is presented to dendritic cells in the form of soluble protein which will be taken into the dendritic cells and processed for MHC/peptide presentation of the cell surface; (4) dendritic cells express B7 on

their surface alleviating the necessity to transfect this cDNA into dendritic cells; (5) the use of endogenous B7 (either B7.1 and/or B7.2) on the dendritic cell surface eliminates the need to provide T cells with IL-2 or other cytokines either in the form of the cytokine itself or transfection of the cDNA into specific cells; (6) all procedures are carried out using the

5 patient's own cells.

DCs obtained as described above, are exposed *in vitro* to a prostate specific antigen, a prostate cancer antigen or an antigenic fragment of either antigen (*e.g.*, PSMA at about 0.1 μ g to about 1000 μ g) in combination with BCG (approximately 2×10^5 to about 1×10^6 Units/ml final concentration) or BCG in combination with LPS (40 Units/ml), washed and administered to a patient to elicit an immune response or to augment an existing immune response. As such, the DCs constitute an anti-prostate cancer vaccine and/or immunotherapeutic agent. DCs presenting a prostate specific antigen are administered to a patient, via intravenous infusion, at a dose of about 10^6 - 10^8 cells. The immune response of the patient can be monitored. Infusion can be repeated at desired intervals based upon the patient's measured immune response.

The following examples demonstrate that human dendritic cells, obtained from prostate cancer patients, pulsed with antigen, for example in the form of autologous tumor lysate or peptide in the presence of a combination of BCG and LPS, or with BCG alone, stimulate antigen-specific cytotoxic T cell and humoral immune responses to human prostate cancer antigens. These examples are presented for purposes of illustration only and are not intended to limit the scope of the invention in any way.

EXAMPLE 1

The following example describes the isolation and culturing of human dendritic cells. Isolated dendritic cells were contacted with tumor cell lysate, and partially purified tumor cell lysate in combination with BCG to demonstrate the stimulation of antigen-specific cytotoxic T cell response.

Culture of Patient Dendritic Cells

Cultures of human DCs were established as described previously herein and in U.S. Patent No. 5,788,963 (incorporated herein by reference). Briefly, peripheral blood mononuclear cells (PBMC) were obtained from leukocyte-enriched "buffy coats" by standard

centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). Plastic-adherent PBMCs (about 1 hour at 37°C) were cultured for 6 to 7 days in AIM-V either supplemented or not with 2% autologous serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM non-essential amino acids, and 1 mM pyruvate (referred to as

5 "culture medium"; all from Boehringer Ingelheim, Biowhittaker, Verviers, Belgium) in the presence of 1000 U/ml or 500 U/ml of each granulocyte macrophage-colony stimulating factor (GM-CSF) (Leucomax™ 1.11 x 10⁷ U/mg from Novartis, Basel, Switzerland) and 500 U/ml Interleukin-4 (IL-4) (Schering-Plough Research Institute, Kenilworth, NJ).

10 **Phenotyping of Patient DCs**

To determine surface antigen (Ag) expression, cells (10⁵ DCs in 50 µl) were labeled with primary monoclonal antibody (mAb) in complete medium followed by FITC-conjugated F(ab')₂ fragments of goat anti-mouse Ig (Dako, Glostrup, Denmark). The following monoclonal antibodies can be used although numerous others having the same specificity are well known: G46-2.6 (IgG₁, anti-HLA-ABC), L243 (IgG_{2a}, anti-HLA-DR), HB-15a (IgG_{2b}, anti-CD83), BU63 (IgG₁, anti-CD86). Washes were in HBSS containing 0.2% albumin. After the last wash, the cells were stored in HBSS containing 0.2% albumin and 2% formaldehyde. The samples were analyzed on a FACScan® (Becton-Dickinson, San Jose, CA). Data was analyzed and presented using CellQuest® software from Becton-Dickinson.

20 **Preparation of Tumor Cell Lysates**

BCG and LPS were used to stimulate MHC-class I loading of DCs as follows: 1-100 µg of LNCaP-derived PSMA or partially purified recombinant PSMA (rPSMA) was 25 added to the culture medium of day 6 DCs by pipetting with a sterile microtip. At the same time, BCG (0.2 - 1.6 x 10⁶ U/ml final concentration; Tice-BCG, Organon Teknika, Durham, NC) and, in a replicate flask, LPS (40 U/ml final concentration) was added to the culture medium using the same method. The culture medium was then mixed gently before being returned to a CO₂ incubator.

30 Osmotic loading was performed as follows: day 7 DCs were harvested by vigorous pipetting with phosphate buffered saline (PBS), then incubated with PBS preceding mechanical dislodging. DCs were centrifuged at low speed, and all supernatant was removed

using aspiration. The cell pellets were resuspended in the desired amount of PSMA and PBS to a final volume of 100 μ l. Subsequently, 100 μ l hypertonic medium (1 M sucrose and 20% glycerol) was added, and the cell suspension mixed with a pipet microtip. The cells were then placed in a 37°C water bath for 10 minutes, in a 15 ml centrifuge tube. Following the 5 incubation, isotonic conditions were restored by filling the tube with DMEM or AIM-V, then returning the tube to the 37°C water bath for an additional 3 minutes. Cells were then centrifuged and resuspended in culture medium at the desired concentration.

Following two weekly stimulations *in vitro* with PSMA loaded DCs and one or more weekly restimulations with PBMC exogenously pulsed with PSMA, specific 10 reactivity to the immunogen was demonstrated (Fig. 1-5). By day 18 of culture, effector cells from Patient 92 specifically recognized autologous DCs presenting PSMA but not the irrelevant protein ovalbumin (OVA) or untreated DCs (Fig. 1A). Patient 92 effector cells also specifically recognized allogeneic DCs from Patient 105 and I.T. (Fig. 1B and Fig. 1C). Effector cells from both Patient 105 and I.T. secreted cytokine in a specific manner following cocultivation with both autologous and allogeneic DCs presenting PSMA.

The observed PSMA-restricted activity contains a significant CD8 $^{+}$ T cell component (Fig. 2A - Fig. 2C). As illustrated, when the effector cells from patient 105 were stimulated using DCs in which PSMA had been loaded in the presence of BCG, cytokine secretion was significantly CD8-mediated, regardless of whether LPS was included during 15 stimulations (Fig. 2A and Fig. 2B). When the effector cells were stimulated using DCs expressing PSMA following osmotic loading, there was not a statistically significant difference in the relative contribution of CD8 $^{+}$ and CD4 $^{+}$ T cells to the observed reactivity (Fig. 2C). Similar findings were obtained using identically stimulated effectors from patients 20 92 and I.T. (data not shown). These results demonstrate that use of BCG, with or without LPS, as presently taught, yields an immune response biased towards cytotoxic T cells (i.e., over 50% of T cells were CD8 $^{+}$).

Following additional restimulations with PSMA-loaded PBMC (using osmotic loading), specific effectors from patient 105 were evaluated to determine whether these 25 effectors had the ability to secrete cytokines in a dose-dependent manner (Fig. 3). For the initial stimulations at days 0 and 10, if BCG or BCG + LPS was present during PSMA loading into DCs the amount of cytokine secreted by the effectors was directly associated with the amount of PSMA osmotically loaded into autologous DC targets (Fig. 3A and 3B).

The greatest amount of IFNy secretion was observed when the highest concentration of PSMA tested was loaded into DCs, without a maximum being reached. All stimulations *in vitro* were performed using DCs or PBMC loaded with 10 µg of PSMA. This amount of protein was sufficient for strong T cell stimulation and activation. When the experiment was 5 repeated with the maximum concentration of PSMA increased to 30 µg, a plateau was achieved (Fig. 3C).

A moderate level of PSMA specific cytotoxicity can be detected (Fig. 4A and 4B). At 32 days of culture, effector cells from Patient I.T. displayed 37% lysis of autologous 10 DCs presenting PSMA at an effector:target ratio of 40:1, as compared with 23% against autologous DCs presenting OVA or 19% against untreated targets (Fig. 4A). At 39 days of culture, effector cells from Patient 92 displayed 23% lysis of autologous DCs 15 presenting PSMA at an effector:target ratio of 36:1, as compared with 14% against autologous DCs presenting OVA or 10% against untreated targets (Fig. 4B).

Specificity of PSMA-derived peptides recognized by T cells stimulated *in vitro* with DCs osmotically loaded with intact, soluble protein was also determined. Using 20 effector cells from Patient 92 that were PSMA-specific following stimulation *in vitro*, cytokine secretion was measured after co-culturing with the antigen-presenting cell line T2 exogenously pulsed with one of the HLA-A2⁺ peptides derived from PSMA:PSM-P1 or influenza M1 protein (Fig. 6A and Fig. 6B).

In an additional experiment it was demonstrated that dendritic cells 25 osmotically or directly loaded with influenza M1 protein or peptide, comprising an antigen fragment and matured in the presence of either BCG alone or in combination with interferon gamma are capable of stimulating a T cell mediated activity as measured by the production of interferon gamma by the V_{β17} T cell subset.

25

EXAMPLE 2

The present example examines whether dendritic cells retain their function after cryopreservation. This characteristic is particularly important because immunotherapy approaches involve multiple treatments and it is preferable that all the DCs for each patient 30 be prepared and loaded with antigen during a single preparation, then aliquoted and cryopreserved for subsequent infusion. It was possible that the freezing and thawing of the DCs may limit their effectiveness as CD8⁺ T cell activators.

Dendritic cells were isolated from PBMC of a prostate cancer patient and cultured, as described above, for 7 days in the presence of 500 U/ml GM-CSF and 500 U/ml IL-4. On day 7, the isolated DC's were harvested and cryopreserved using 90% fetal calf serum and 10% dimethylsulfoxide. The cryopreserved DC's were subsequently stored frozen 5 for a period of time, thawed in a 37°C water bath and transferred to a 15 ml polypropylene tube and centrifuged at 1200 rpm for 5 min. The thawed DC's were then resuspended in medium containing 10% heat-inactivated human serum and counted and used as described below.

Day 7 DCs from Patient 105 were osmotically loaded with PSMA or OVA (or 10 left untreated). The treated DCs were then frozen in a standard freeze medium of 90% human serum and 10% DMSO as described, *supra*. On the same day, a second set of DC cultures was established. One week later, fresh day 7 DCs were harvested and osmotically loaded with PSMA or OVA (or left untreated). In addition, DCs that had been cryopreserved 15 the week before were thawed by standard techniques and used immediately in an ELISA. PSMA-restricted effector cells from Patient 105 displayed strong reactivity to both fresh and cryopreserved targets (Fig. 5). As taught in this specific example, cryopreservation does not diminish the effectiveness of DCs to function as antigen presenting cells.

Inciting a potent anti-tumor response using immunotherapy has been limited in 20 efficacy partly due to difficulty in stimulating a cytotoxic T cell response. The present invention describes methods and compositions to overcome this limitation. The invention entails exposing dendritic cells to soluble tissue specific antigen in the presence of Bacille Calmette Guerin (BCG), such that the BCG helps direct the antigen into the MHC-class I processing pathway inducing a predominantly cytotoxic T cell response. Recent work by Cella and colleagues (Cella et al., *Nature* 388:782-787 (1997)), has also demonstrated the 25 importance of the maturation process for antigen presentation. In the absence of inflammatory stimuli, the half life of an antigen presented on MHC-class II molecules on DCs was 10 hours. In contrast, pulsing the DCs with antigen in the presence of inflammatory factors increased the half life of the antigen to 100 hours. The longer half life allows the DCs to home to secondary lymphoid organs and to activate antigen-specific T-lymphocytes.

EXAMPLE 3

In this example dendritic cells isolated from a cancer patient were isolated and treated with various concentrations of BCG. After several days of culture, the DCs were tested for 1) the capacity to uptake particles by pinocytosis, and 2) the surface expression of 5 certain dendritic cell maturation markers, including HLA-DR, CD86, CD40, CD83, CD80 and HLA-class I.

Dendritic cells were isolated from patient 57 as described above. The isolated cells ($1-5 \times 10^6$) were cultured for about 6 days in eight T-75 flasks. BCG (1×10^6 units/ml) was added to duplicate flasks to achieve a dilution of 1:250, 1:2,500, or 1:25,000. No BCG 10 was added to the two remaining culture flasks. A first set of culture flasks comprising DCs with no BCG, or with 1:250; 1:2,500; or 1:25,000 BCG dilution was harvested after a 48 or 72 hour incubation. The duplicate set of culture flasks was harvested after a total of 72 hours in culture. Each DC culture was analyzed for: (1) the capacity to uptake FITC/Dextran by pinocytosis, and (2) the level of surface expression of particular DC maturation markers, including HLA-DR, CD86, CD40, CD83, CD80, and HLA-class I.

The capacity to uptake labeled dextran particles by pinocytosis was tested as follows: Each DC culture flask contents was aliquoted into duplicate tubes and incubated in AIM-V medium on ice for 30 minutes. Following incubation on ice, FITC/Dextran was added to each tube to achieve a final concentration of approximately 1 mg/ml. One set of tubes was incubated at 37°C, in 5% CO₂, for 1 hour, while the duplicate set of tubes was incubated on ice (0°C) for approximately 1 hour. DCs were washed 3 times in phosphate buffered saline (PBS) prior to analysis by flow cytometry. The relative amount of pinocytosis by DCs was compared after subtracting background uptake at 0°C (Table 2).

For analysis of DC maturation markers each DC culture was incubated with 25 the following monoclonal antibody pairs: FITC-anti HLA-DR/PE-anti-CD86; FITC-anti CD40/PE – anti-CD83; FITC-anti-CD80/PE-anti-HLA-class I; or FITC-/PE-isotype antibody controls using standard methods. Surface expression of each DC markers were analyzed by flow cytometry (Table 3).

Table 2

FITC – Dextran Uptake (Mean Fluorescence 37°C – Mean Fluorescence 0°C)

Exp. No.	BCG concentration	Duration (h)	Mean	GEO Mean	Median
1	0	48	51	36.3	39.15
2	1:250	48	28.09	20.76	22.03
3	1:2500	48	37.02	27.11	29.28
4	1:25000	48	40.44	34.12	38.04
5	0	72	63.8	43.2	47.3
6	1:250	72	34.76	22.85	22.6
7	1:2500	72	49.79	36.24	39.6
8	1:25000	72	55.62	38.27	41.41

Table 3

Mean FL difference

Exp. No.	BCG concentration	Duration (h)	HLA-DR FITC	CD86 PE	CD40 FITC	CD83 PE	CD80 FITC	HLA-Class I PE
1	0	48	250	613.07	19.64	15.14	-1.1	140.28
2	1:250	48	276.54	957.38	24.78	62.58	-1.27	173.69
3	1:2500	48	267.94	734.1	21.9	21.4	-0.94	135.45
4	1:25000	48	277.1	632	18.2	17.59	-1.62	148.97
5	0	72	337.13	705.75	20.89	22.42	-1.26	193.06
6	1:250	72	331.71	1106.12	33.39	127.65	-2.11	373.06
7	1:2500	72	336.87	694.74	24.2	35.1	-1.41	177.6
8	1:25000	72	298.96	646.95	19.81	24.25	-1.15	170.26

5
.....

At a concentration of 1:250 DCs showed a significant increase in CD86, CD40, CD83 and HLA-class I in both the 48 and 72 hour cultures. The effect of BCG was more pronounced at 72 hours. For example, HLA-class I increased by 24 % after 48 hours in 1:250 BCG, but increased 93 % after 72 hours of 1:250 BCG. Likewise, CD83 increased by 40 fold at 48 hours in 1:250 BCG and 5.7 fold after 72 hours in 1:250 BCG.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims. The scope of the invention should, therefore, be determined not with reference to the above 5 description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

10